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SHORT COMMUNICATION

A Temperature-Sensitive Mutation of the Vaccinia Virus E11 Gene Encoding a 15-kDa Virion Component

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The *ts*49 mutation of vaccinia virus WR was mapped by marker rescue to the E11 gene encoding a 15-kDa polypeptide. During synchronous infection of BSC40 cells with wild-type virus, immunoreactive E11 protein accumulated in parallel with the onset of late protein synthesis. Immunoblotting of extracts of wild-type virions showed that the E11 protein was encapsidated within the virus core. A normal temporal pattern of viral protein synthesis was observed in cells infected with *ts*49 at the nonpermissive temperature (40°) and normal-appearing *ts*49 progeny virions were observed by electron microscopy. Sequencing of the E11 gene of *ts*49 revealed a single amino acid substitution, Gly(66)Arg, in the mutant E11 polypeptide. The steady-state level of E11 protein during *ts*49 infection was much lower than that observed during infection with wild-type virus. This was the case at both the permissive and nonpermissive temperatures. We discuss two possible explanations for the thermosensitive growth of *ts*49: (i) that virus infectivity requires a threshold level of active E11 protein or (ii) that E11 function is conditionally essential.

Genes essential for the replication of vaccinia virus in cell culture have been identified by classical genetic approaches involving the isolation and characterization of temperature-sensitive mutant viruses (*1*). A collection of 72 *ts* mutants of vaccinia strain WR has been assigned to 32 genetic complementation groups by Condit and co-workers (*2*). At least one isolate from each of the complementation groups has been analyzed with respect to the pattern of viral protein and DNA synthesis under nonpermissive growth conditions (*3, 4*). Mutants in 22 complementation groups exhibit a so-called "normal" phenotype, i.e., they display a wild-type pattern of DNA replication and protein synthesis at the nonpermissive temperature.

The normal phenotype is suggestive of mutation of a viral gene encoding a protein that functions in virion assembly or in establishing the next round of infection, for example, during the synthesis of early mRNAs by the virion-encapsidated RNA polymerase. This notion has been borne out by the mapping of *ts* mutations to individual vaccinia genes. Several normal mutations affect enzymatic components of the virus particle with a role in early transcription. Examples include the H4 protein, a subunit of the virion RNA polymerase (*5, 6*); the D6 protein, a subunit of the early transcription initiation factor (*7*); and the I8 protein, an RNA helicase (*8, 9*). At the nonpermissive temperature, these mutations result in the production of microscopically normal-appearing virions that are noninfectious. Other normal *ts* mutations also map to constituent polypeptides of the virion. These include the D2, D3, and I7 proteins (*10, 11*), for which no enzymatic activity has yet been demonstrated, and the F10 protein, which is a serine–threonine protein kinase (*12, 13*). Mutations of each of these proteins result in defective assembly of new virus particles at the nonpermissive temperature.

There are nine complementation groups of normal *ts* mutants from the Condit collection that have not yet been mapped to specific vaccinia genes. In this paper, we present a molecular genetic and phenotypic analysis of the vaccinia *ts*49 mutant, the lone member of complementation group 8 (*2*). *ts*49 was obtained from R. Condit and subjected to two rounds of plaque purification in BSC40 cells grown at 31° (permissive temperature) prior to amplification in monolayer cultures at 31°. Stocks of *ts*49 were tested for their ability to replicate in BSC40 cells at 31 and 40°. Titration experiments showed that plaque formation by wild-type virus was equivalent at 31 and 40°. In the case of *ts*49, the ratio of plaques formed at 40 to 31° was 1/10,000. A one-step growth experiment was performed with wild-type and *ts*49 viruses at both 31 and 40°. Cells were harvested at 2, 12, 24, and 48 hr after infection, and the production of infectious virus was assayed by titration at 31° (Table 1). The yield of *ts*49 progeny at the permissive temperature was about half of that of wild-type virus at each time point. No increase in the titer over the 2-hr background was observed when infection with *ts*49 was performed at the nonpermissive

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TABLE 1

One-Step Growth of *ts*49 Virus at Permissive and Nonpermissive Temperatures

Virus	Temperature	Titer (PFU/ml) 2 hr	12 hr	24 hr	48 hr
WT	31°	2×10^5	1×10^7	2×10^7	1×10^8
	40°	2×10^4	1×10^7	3×10^7	1×10^8
*ts*49	31°	3×10^5	4×10^6	1×10^7	3×10^7
	40°	3×10^5	2×10^5	2×10^5	3×10^5

Note. BSC40 cells maintained at 31 or 40° were infected with WT or *ts*49 virus at an m.o.i. of 10. At the indicated times postinfection, cells were harvested and titered by serial 10-fold dilution onto BSC40 cells at 31°. Plaques were visualized at 2 days postinfection by staining with 0.1% crystal violet in 20% ethanol.

temperature. Growth of wild virus did not vary with temperature, as expected. Thus, the plaque phenotype of the *ts*49 mutant was clearly attributable to a thermosensitive defect in virus replication.

Viral protein synthesis was analyzed by pulse-labeling synchronously infected cells with [^{35}S]methionine. Cells infected at a multiplicity of 10 with wild-type or *ts*49 virus at 31° displayed the typical developmental pattern of vaccinia gene expression, i.e., the appearance of novel early polypeptides at 2–4 hr postinfection, transition to the synthesis of distinctive late proteins by 8 hr (these include the major structural protein precursors p4a and p4b), and shut-off of host protein synthesis late in infection (Fig. 1, top). Identical transitions were observed in cells infected with *ts*49 virus at 40°; however, the onset of late viral protein synthesis was accelerated relative to that at 31° (Fig. 1, bottom). There was no apparent difference between the protein synthesis profiles of wild-type and *ts*49 virus-infected cells at either growth temperature (Fig. 1).

Virus morphogenesis was evaluated by electron microscopy of *ts*49-infected cells. Spherical immature particles and brick-shaped intracellular mature virions (IMV) were evident in the cytoplasm at 24 hr postinfection. Spherical and IMV forms were also observed in *ts*49-infected cells at the nonpermissive temperature (not shown). *ts*49 IMV particles formed at 40° were microscopically similar to wild-type IMV. Aside from sporadic "half-moon" spherical particles observed at 40° (*11*), we found no evidence for the accumulation of aberrant viral structures at either growth temperature.

It was reported previously that *ts*49 could be restored to temperature-independent growth by marker rescue with a plasmid containing the entire *Hin*dIII E genomic fragment derived from wild-type virus (*2*). An annotated map of the 15.2-kb E fragment, which includes 12 viral genes (*14*), is shown in Fig. 2. We mapped the *ts*49 mutation more precisely using an electroporation-based method for DNA-mediated marker rescue (*6*, *11*). Cells infected with *ts*49 were electroporated with plasmid DNA and plated onto uninfected cell monolayers maintained at the nonpermissive temperature. Successful marker rescue yielded recombinant wild-type progeny from individual electroporated cells that were seeded onto the virgin monolayer; this was manifested as a plaque arising

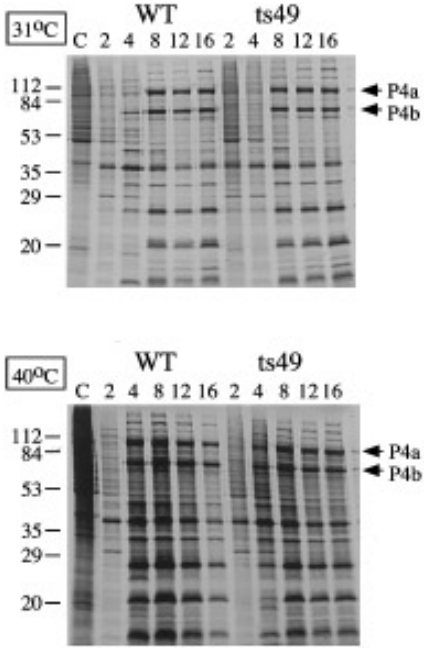

FIG. 1. Synthesis of viral proteins by *ts*49 during synchronous infection. Confluent BSC40 monolayers were infected with virus at an m.o.i. of 10 at 31° (top) or 40° (bottom). The inoculum was removed after 30 min, and cells were washed once with DME medium and then overlaid with fresh DME. At various times thereafter, the medium was removed, and cells were washed with methionine-free DME and then overlaid with methionine-free DME containing 30 μCi of [^{35}S]methionine per milliliter (>800 Ci/mmol). After pulse-labeling for 30 min at 31 or 40°, the medium was removed and cells were lysed *in situ* by the addition of 0.15 ml of a solution containing 0.065 *M* Tris–HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol. Aliquots (15 μl) were heated at 100° for 5 min and then electrophoresed through a 12% polyacrylamide gel containing 0.1% SDS. Autoradiographs of the gels are shown. The time at which the pulse-labeling was initiated (hours after removal of the virus inoculum) is indicated above each lane. The identity of the infecting virus is noted above each set of time points. Uninfected cells were pulse-labeled in parallel as a control (lane C). The positions and sizes (in kDa) of prestained marker proteins are indicated to the left. Two abundant viral late proteins, corresponding to virion precursors p4a and p4b, are denoted by arrows to the right.

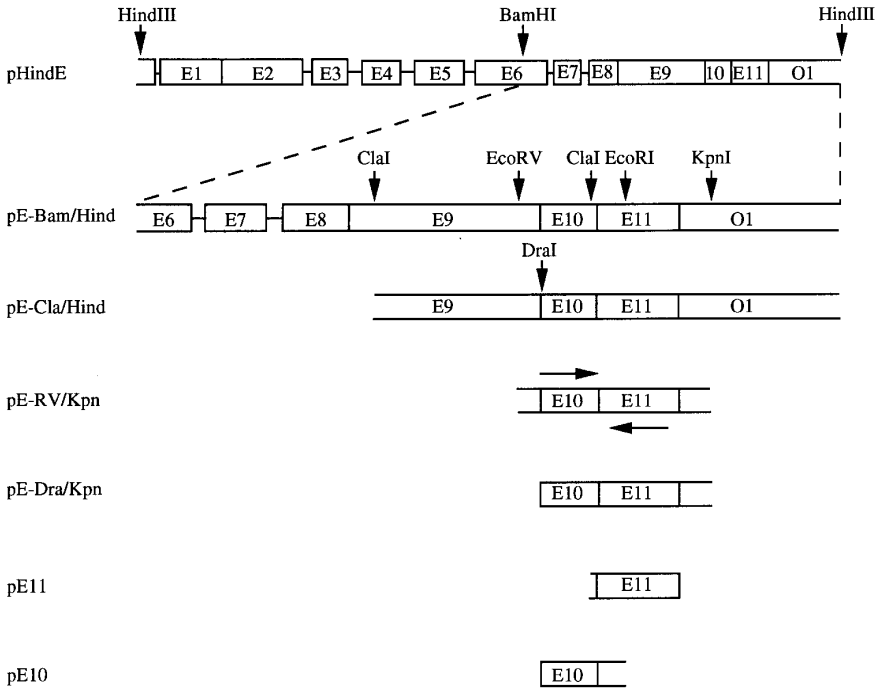

FIG. 2. Map of the genomic *Hin*dIII E fragment and subfragments used for marker rescue. Plasmid pHind-E contains the full 15.2-kb *Hin*dIII E fragment of vaccinia WR cloned into pBR322 (*21*). A physical and genetic map of the viral DNA insert is shown. Protein encoding regions of the E fragment (illustrated as boxed segments) are distinguished from noncoding regions (intervening line segments). Plasmid pE-Bam/Hind (*21*) contains a 7.2-kb segment of the E fragment extending from the internal *Bam*HI site to the right terminal *Hin*dIII site; an expanded map of this insert is shown below that of the E fragment. Plasmid pE-Cla/Hind contains 5.4 kb of DNA from the right end of the E fragment (*21*). Other subclones were constructed by insertion of gel-purified restriction fragments into the polylinker of pBS(−) (Stratagene). Restriction sites used in subcloning are indicated by vertical arrows. Plasmids were named according to the restriction sites at the borders of the viral insert. Genes E10 and E11 are oriented in a tail-to-tail fashion, with E10 transcribed in a rightward direction and E11 transcribed in the leftward direction. A DNA fragment containing both the E10 and E11 genes was PCR-amplified from pHind-E using flanking oligonucleotide primers containing restriction sites for *Nde*I and *Nco*I, respectively. The PCR reaction product was gel-purified and then cleaved into E10- and E11-containing fragments by treatment with *Nde*I and *Eco*RI or with *Nco*I and *Cla*I. A 386-bp *Nde*I–*Eco*RI fragment containing the entire E10 coding sequence and a 100-bp segment from the 3′ end of the E11 gene was gel-purified and inserted into pET14b (Novagen) to generate pet-E10. A 409-bp *Nco*I–*Cla*I fragment containing the entire E11 ORF and 25 bp from the 3′ end of the E10 gene was purified and inserted into plasmid pET14b to generate pet-E11. The viral inserts were then transferred to pBS(−) vector backgrounds. A *Bgl*II/*Eco*RI fragment was excised from pet-E10 and inserted into pBS(−) cut with *Bam*HI/*Eco*RI to yield pE10. An *Sph*I/*Cla*I fragment of pet-E11 was inserted to into pBS(−) cut with *Sph*I/*Acc*I to yield plasmid pE11. DNAs used for marker rescue experiments were prepared by alkaline lysis and purified using the Wizard Maxipreps DNA purification system (Promega).

from each infectious center. No infectious centers were observed when monolayers were seeded with *ts*49-infected cells that had been electroporated without added DNA (Fig. 3). Marker rescue was achieved by electroporation with the pHind-E plasmid containing the entire wild-type *Hin*dIII E fragment, but not with plasmid pHind-F containing the 13.2-kb wild-type *Hin*dIII F fragment (Fig. 3). Rescue was also obtained with a *Bam*HI/*Hin*dIII subclone corresponding to the right half of the E fragment and including genes E6 to O1, with a *Cla*I/*Hin*dIII subclone containing genes E9 to O1, and with *Eco*RV/*Kpn*I and *Dra*I/*Kpn*I subclones that included only genes E10 and E11 and a portion of the O1 gene (Fig. 3). Final assignment of the *ts*49 mutation was made by amplifying the wild-type E10 and E11 coding sequences by PCR and cloning them separately into pBS vectors. Plasmid pE11 containing the complete E11 gene and only 25 bp of sequence from the E10 gene afforded marker rescue, whereas a plasmid containing the entire E10 gene and a 3′ segment of the E11 gene did not (Fig. 3). We concluded, therefore, that the genetic lesion of *ts*49 localized to the E11 gene.

The nucleotide sequence of the wild-type E11 gene of the WR strain of vaccinia was identical to the published sequence of the E11 gene of the Copenhagen strain (*14*). The E11 gene encodes a protein of 129 amino acids with a predicted molecular mass of 14.9 kDa. The molecular lesion of the *ts*49 virus was determined by sequencing individual plasmid clones of the *ts*49 E11 gene obtained by PCR amplification of cytoplasmic DNA isolated from *ts* virus-infected cells. Four different E11 clones were sequenced for *ts*49, each of which contained a single coding change (GGG to AGG) resulting in a Gly to Arg substitution at position 66.

The DNA sequence of the E11 gene suggested that it is would likely be expressed at late times during vaccinia infection. For example, the ATG codon of the E11 protein is situated within a TAAATG sequence; the TAAAT motif is an essential component of a vaccinia late promoter, and the close proximity of the TAAAT to the translation

start site is common feature of late genes (*15*). Furthermore, the E11 gene contains two internal TTTTTNT motifs that specify termination of early gene transcription. Such motifs tend to be excluded from the coding sequence of viral early genes, but are usually detected with late coding sequences because this signal is not recognized as a terminator during late transcription (*16*).

The accumulation of E11 protein during synchronous infection of BSC40 cells with wild-type vaccinia virus was examined by Western blotting with anti-E11 serum (Fig. 4). The antibody was raised in rabbits immunized with His-tagged E11 protein that had been expressed in bacteria and purified to near-homogeneity from soluble bacterial lysates under nondenaturing conditions (S. Wang and S. Shuman, unpublished). Whole-cell lysates of virus-infected cells were resolved by SDS–PAGE; the proteins were transferred to membranes, which were probed with the anti-E11 serum and visualized by ECL. A 15-kDa immunoreactive polypeptide was detectable at 4 hr postinfection at 31°; this species increased in abundance by 8 hr and persisted at 12 and 16 hr postinfection (Fig. 4). No such immunoreactive polypeptide was detected in uninfected cells (Fig. 4, lane C). As anticipated, the immunoreactive E11 protein made during virus infection migrated more rapidly during SDS–PAGE than the His-tagged E11 polypeptide antigen used to prepare the anti-

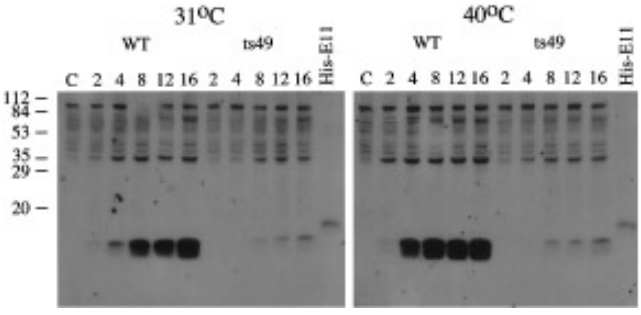

FIG. 4. Expression of the E11 protein during virus infection. BSC40 monolayers were infected synchronously at 31° (left) or 40° (right) with wild type (WT) or *ts*49 virus. At the indicated times (hr) after removal of the inoculum, the cells were lysed *in situ* as described for the metabolic labeling experiments. Lysates (30-μl aliquots) were heated at 100° for 5 min and then electrophoresed through a 15% polyacrylamide gel containing 0.1% SDS. Polypeptides were transferred electrophoretically to an ECL nitrocellulose membrane (Amersham) that was then blocked in TBST buffer (10 m*M* Tris–HCl, pH 8.0, 150 m*M* NaCl, 0.1% Tween-20) containing 10% dry milk. Membranes were incubated for 1 hr at room temperature with anti-E11 rabbit serum diluted 1:4000 in TBST. After removal of serum and washing with TBST, bound antibodies were localized by incubation with HRP-conjugated secondary antibody using an ECL detection system according to the instructions of the manufacturer (DuPont NEN). The two blots shown in the figure were exposed simultaneously to film. The identity of the infecting virus is noted above each set of time points. Lysates of uninfected cells were analyzed in parallel as a control (lane C), as was a sample of recombinant His-E11 protein (His-E11). The positions and sizes (in kDa) of prestained protein markers are indicated to the left.

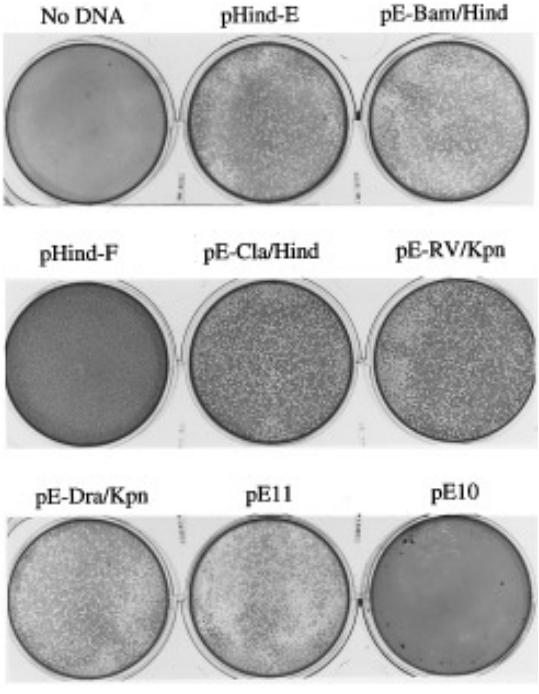

FIG. 3. Marker rescue of *ts*49. Electroporation-based marker rescue using supercoiled plasmid DNAs was performed as described (*6, 11*). After 2 days of incubation at 40°, the cells were stained with 0.1% crystal violet in order to visualize plaques formed from infectious centers. Photographs of the stained monolayers are shown. The plasmid tested in each marker rescue is indicated above the well. The "No DNA" control entailed seeding the monolayers with virus-infected BSC40 cells that had been electroporated without added DNA.

serum (Fig. 4, lane His-E11). Note that the infected cell lysates analyzed by Western blotting were the very same ones used in the metabolic labeling experiment shown in Fig. 1. Hence, we can conclude that the appearance of the immunoreactive E11 protein coincided with the onset of late protein synthesis. Accumulation of E11 protein in cells infected with wild-type virus at 40° was of earlier onset than that at lower temperature, consistent with the pulse-labeling experiment showing that the transition to late protein synthesis was accelerated during infection at 40°.

Parallel analysis by Western blotting of cells infected with *ts*49 revealed a much lower accumulation of E11 protein compared to that of wild-type virus-infected cells. Remarkably, this was the case at *both* permissive and nonpermissive growth temperatures (Fig. 4). Again, because the samples analyzed were the same ones shown in Fig. 1, we can be confident that the lack of E11 protein accumulation was not secondary to a global failure to synthesize late viral proteins. This result raises interesting questions about the basis for the temperature-sensitive phenotype of *ts*49 that will be considered below.

Vaccinia genes that bear the "normal" class of *ts* mutations often encode proteins that are encapsidated in the virus particle. In order to examine whether the E11 protein is a virion component, we probed virion extracts by Western blotting with anti-E11 serum. The polypeptide compositions of the virion envelope fraction (consisting of material solubi-

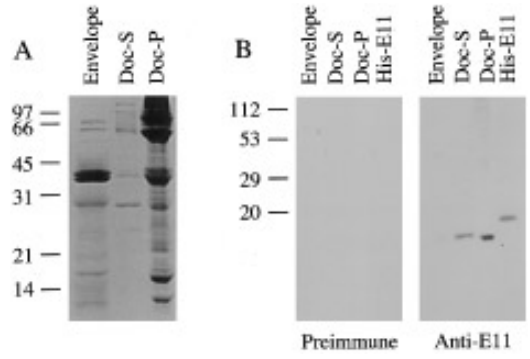

FIG. 5. Association of E11 protein with virus cores. (A) Extracts of purified wild-type virions were prepared as described (*6, 11*). The envelope fraction refers to material solubilized by treatment of virions with 50 m*M* DTT and 0.5% NP-40. The virus core was extracted with buffer containing 0.2% sodium deoxycholate (DOC). Material solubilized by this treatment is referred to as the DOC supernatant (DOC-S) fraction. The residual insoluble material is referred to as the DOC pellet (DOC-P). Aliquots (5 μl) of each fraction were analyzed by electrophoresis through a 15% polyacrylamide gel containing 0.1% SDS. Polypeptides were visualized by staining with Coomassie blue. (B) Aliquots (5 μl) of each virion fraction were electrophoresed through a 15% polyacrylamide gel containing 0.1% SDS. A sample of recombinant His-E11 protein was analyzed in parallel. Polypeptides were transferred to nitrocellulose membranes for immunoblotting with 1:4000 dilutions of either preimmune or anti-E11 sera. Immunoreactive protein was detected by ECL. The two blots shown in the figure were exposed simultaneously to film. The source of the protein fraction is indicated above each lane. The location and sizes (in kDa) of marker proteins are shown to the left of each panel.

lized by treatment with NP-40 and dithiothreitol), a deoxycholate extract of virion cores (0.2% DOC supernatant), and the insoluble pellet fraction (DOC pellet) were examined by SDS–PAGE in order to monitor the integrity of the extraction procedure (Fig. 5A). Virion proteins were partitioned in characteristic fashion. Western blotting revealed immunoreactive E11 protein in the DOC extract of virus cores and in the insoluble core fraction, but not in the envelope fraction (Fig. 5B). No reactive protein of this size was detected when immunoblotting was performed with preimmune rabbit serum. As expected, the core-associated E11 protein migrated more rapidly during SDS–PAGE than the recombinant E11 protein containing the His tag (Fig. 5B). These data indicate that the E11 protein is a component of the vaccinia core.

In summary, we find that a single amino acid substitution in the E11 protein accounts for the conditional growth phenotype of *ts*49. The E11 gene product is a 15-kDa protein that accumulates at late times during infection with wild-type virus and becomes encapsidated within the core of the infectious virion. In agreement with Condit's original observations (*4*), we found the pattern of viral protein synthesis in *ts*49-infected cells to be indistinguishable from that of wild-type virus, at least at the level of resolution afforded by SDS–PAGE analysis of [^{35}S]methionine-labeled proteins from whole-cell lysates. We determined by electron microscopy that normal-appearing IMV particles were present in the cytoplasm of *ts*49-infected cells at the nonpermissive temperature.

The formation of microscopically normal but noninfectious IMV particles has been documented for other temperature-sensitive or conditional null mutations affecting constituents of the virus core. These include the H4 subunit of the virion RNA polymerase (*6, 17*), the I8 RNA helicase (*8*), the D6 subunit of the vaccinia early transcription factor (*7*), and the L4 DNA binding protein (*18*). In addition, the Dales collection of *ts* mutants of vaccinia IHD includes four isolates (category Q) that form mature particles of normal appearance at the nonpermissive temperature (*19*). In only a few cases has the lack of infectivity been correlated with a specific biochemical defect of the progeny particles, for example, the failure to encapsidate sufficient RNA polymerase in the case of conditional mutants in the H4 and D6 genes (*17, 7*). Additional insights into the role of the E11 protein in virus infectivity should emerge once the *ts*49 virions assembled at the nonpermissive temperature are purified and subjected to detailed biochemical analysis.

The finding that the level of E11 protein expression is much lower in *ts*49-infected cells at both permissive and nonpermissive temperatures raises intriguing questions about the basis for the conditional growth phenotype. We attribute the low steady-state level of *ts*49 E11 protein to the point mutation described herein. Formally, this can be secondary to a decreased rate of E11 protein synthesis (either because of impaired translation or lower steady-state levels of E11 mRNA) or an enhanced rate of E11 protein turnover. Although we have not measured these parameters directly, we favor the latter hypothesis. Studies of other vaccinia *ts* mutants affecting the D2, D3, and B1 genes provide ample precedent for an enhanced rate of specific degradation of the mutated protein at both nonpermissive and permissive temperatures (*10, 20*).

Normal replication of *ts*49 at permissive temperature despite the very low steady-state levels of E11 protein argues that the amount of E11 made by wild-type virus is in excess of what is required for virus infectivity, at least at 31°. Yet, this same low amount of E11 protein clearly does not suffice at 40°. Does this reflect a temperature-sensitive defect in the *ts*49 E11 protein, or does the problem reside elsewhere? If the residual *ts*49 E11 protein is functionally temperature-sensitive per se, then its activity would be reduced to a subthreshold level at the nonpermissive temperature. There is precedent in the case of the F10 protein kinase for temperature-sensitive replication of vaccinia virus being correlated with thermolabile enzymatic activity *in vitro* (*13*). Obviously, this issue is difficult to evaluate in the present case without a demonstrable biochemical activity for the E11 gene product. An alternative explanation of the conditional growth defect is that the level of active *ts*49 E11 protein does not vary with temperature, but rather the threshold level of E11 required for infectivity is increased at 40°. In this case, it might be predicted that growth at 40° would be

restored if the level of *ts*49 E11 protein were increased to a higher set point. Yet another model is that the *ts*49 mutation is functionally equivalent to a null mutation, either because the mutant protein is truly inactive or because E11 protein level drops below a putative threshold at either growth temperature. In this view, functional thermolability would be attributed to a second essential protein, either virus-encoded or host-encoded, that complements E11 function at permissive temperature, but not at nonpermissive temperature. This has been suggested previously for *ts* mutations of the vaccinia B1 protein kinase (*20*). Future experiments with *ts*49 will be aimed at testing these models, specifically by attempting to delete the E11 gene and, if this is achieved, by determining whether the deletion virus displays a temperature-sensitive phenotype.

REFERENCES

1. Condit, R. C., and Niles, E. G., *Curr. Top. Microbiol. Immunol.* 163, 1–35 (1990).
2. Thompson, C. L., and Condit, R. C., *Virology* 150, 10–20 (1986).
3. Condit, R. C., and Motyczka, A., *Virology* 113, 224–241 (1981).
4. Condit, R. C., Motyczka, A., and Spizz, G., *Virology* 128, 429–443 (1983).
5. Ahn, B., and Moss, B., *Proc. Natl. Acad. Sci. USA* 89, 3536–3540 (1992).
6. Kane, E. M., and Shuman, S., *J. Virol.* 66, 5752–5762 (1992).
7. Li, J. M., Pennington, J., and Broyles, S. S., *J. Virol.* 68, 2605–2614 (1994).
8. Fathi, Z., and Condit, R. C., *Virology* 181, 273–276 (1991).
9. Shuman, S., *Proc. Natl. Acad. Sci. USA* 89, 10935–10939 (1992).
10. Dyster, L. M., and Niles, E. G., *Virology* 182, 455–467 (1991).
11. Kane, E. M., and Shuman, S., *J. Virol.* 67, 2689–2698 (1993).
12. Lin, S., and Broyles, S. S., *Proc. Natl. Acad. Sci. USA* 91, 7653–7657 (1994).
13. Wang, S., and Shuman, S., *J. Virol.* 69, 6376–6388 (1995).
14. Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P., and Paoletti, E., *Virology* 179, 247–266 (1990).
15. Davison, A. J., and Moss, B., *J. Mol. Biol.* 210, 771–784 (1989).
16. Moss, B., *Annu. Rev. Biochem.* 59, 661–688 (1990).
17. Zhang, Y., Ahn, B., and Moss, B., *J. Virol.* 68, 1360–1370 (1994).
18. Wilcock, D., and Smith, G. L., *Virology* 202, 294–304 (1994).
19. Dales, S., Milanovitch, V., Pogo, B. G. T., Weintraub, S. B., Huima, T., Wilton, S., and McFadden, G., *Virology* 84, 403–428 (1978).
20. Rempel, R. E., and Traktman, P., *J. Virol.* 66, 4413–4426 (1992).
21. Traktman, P., Sridhar, P., Condit, R. C., and Roberts, B. E., *J. Virol.* 49, 125–131 (1984).